Opposite Effects of Wortmannin and 2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one Hydrochloride on Toll-Like Receptor-Mediated Nitric Oxide Production: Negative Regulation of Nuclear Factor-κB by Phosphoinositide 3-Kinase

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ABSTRACT

A number of previous studies have suggested the involvement of phosphoinositide 3-kinase (PI3K) in Toll-like receptor (TLR) signaling. However, there have also been a number of conflicting reports. The PI3K inhibitor wortmannin greatly enhanced TLR-mediated inducible nitric-oxide synthase (iNOS) expression and cytokine production in the mouse macrophage cell line Raw264.7. The effect of wortmannin was common to TLR2, -3, -4, and -9 and was accompanied by activation of nuclear factor-κB and up-regulation of cytokine mRNA production. We were surprised to find that another PI3K inhibitor, LY294002, strongly suppressed the production of iNOS and cytokines. This effect of 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4- one hydrochloride (LY294002) was based on its inhibitory effect on mRNA synthesis. Expression of dominant-negative mutants of PI3K in macrophages augmented the lipopolysaccharide-induced expression of iNOS. Introduction of a pH1 vector producing short hairpin RNA that targets a catalytic subunit of PI3K (p110β) also enhanced the TLR-mediated responses. Thus, the augmentation of TLR signals by wortmannin was mediated through the inhibition of PI3K, whereas the effect of LY294002 was not explained by its effect on PI3K. These discrepancies in the effects of pharmacological inhibitors in TLR-signaling may have caused confusion regarding the role of PI3K in innate immunity.

Innate immune reactions are triggered through Toll-like receptors (TLRs) that recognize a variety of microbial products, such as lipopolysaccharide (LPS), peptidoglycans, flagellin, and unmethylated CpG motifs in bacterial DNA (Akira, 2003; Akira and Takeda, 2004). Recognition leads to the production of various cytokines and nitric oxide (NO). The mammalian family of TLRs has now been expanded to 11 members (Akira, 2003; Akira and Takeda, 2004). TLR signaling begins from the Toll-interleukin-1 receptor (TIR)-domain, a motif of ~200 amino acids found in the cytosolic regions of all TLRs and the IL-1 receptor. Adaptor molecules, which also contain the TIR domain, bind to the TIR domain of TLRs because of TIR/TIR interaction on stimulation (Akira, 2003; Akira and Takeda, 2004). At present, the TLR adaptor family consists of four members: MyD88, Toll/IL-1R domain-containing adapter protein/Mal, TICAM-1/TRIF, and TICAM-2/TRAM. Studies in mice lacking each of these individual adaptors have revealed their specific roles. MyD88 is shared by all TLRs except for TLR3, whereas Toll/IL-1R domain-containing adapter protein/Mal binds to TLR2 and TLR4 (Yamamoto et al., 2004). Likewise, TICAM-1/TRIF binds to TLR3 and TLR4, whereas TICAM-2/TRAM binds only to TLR4 (Oshiumi et al., 2003; Yamamoto et al., 2003). The diversity of the adaptor molecules may account, at least in part, for the variety of inflammatory responses to different ligands.

Mammalian PI3K can be grouped into three major classes based on primary sequence, mechanism of regulation, and

ABBREVIATIONS: TLR, Toll-like receptor; PI3K, phosphoinositide 3-kinase; iNOS, inducible nitric-oxide synthase; shRNA, short hairpin RNA; poly(I:C), polyinosinic-polycytidylic acid; CpG DNA, nonmethylated CpG oligodeoxynucleotide; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; NF-κB, nuclear factor κB; LPS, lipopolysaccharide; TIR, Toll-interleukin-1 receptor; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; RT-PCR, reverse transcription-polymerase chain reaction; Malp-2, macrophage-activating lipopeptide-2 kDa; PAGE, polyacrylamide gel electrophoresis; IFN, interferon; pAkt, phosphorylated Akt; TRIF, Toll-interleukin-1 receptor domain-containing adaptor inducing interferon-β; TRAM, Toll-interleukin-1 receptor domain-containing adaptor inducing interferon-β-related adaptor molecule; TICAM, Toll-interleukin 1 receptor domain-containing adaptor molecule.

substrate specificity (Wymann and Pirola, 1998). Class Ia PI3Ks are heterodimers consisting of a catalytic subunit (p110) and a regulatory subunit (p85). These enzymes are believed to be the major source of PI3, -4, and -5-P_3 in vivo on activation of receptor-type protein tyrosine kinases. Mammals have multiple isoforms of class Ia PI3K (Fruman et al., 1998). Different genes encode class Ia catalytic subunits, termed p110α, p110β, and p110δ, whereas two genes encode the associated regulatory subunits, termed p85α and p85β. Targeted disruption of either p110α or p110β causes death in the early stages of embryonic development. Likewise, targeted disruption of all p85α and p85β gene products is lethal (Brachmann et al., 2005). The regulatory p85α and p85β can compensate for each other because mice lacking either p85α or p85β have been generated successfully (Wymann et al., 2003).

There is increasing evidence of the involvement of PI3K in TLR signaling (Fukao and Koyasu, 2003). Binding of tyrosine-phosphorylated TLR2 (Arbibe et al., 2000), TLR3 (Sarkar et al., 2004), MyD88 (Ojaniemi et al., 2003), and TRIF (Aksoy et al., 2005) to PI3K has been reported to cause activation of this lipid kinase and its downstream target, protein kinase B/Akt. However, there have been conflicting reports on the physiological consequences of this phenomenon. The inhibition of PI3K was reported to result in the inhibition of TLR2-mediated activation of NF-κB (Arbibe et al., 2000) or TLR3-mediated activation of interferon regulatory factor-3 (Sarkar et al., 2004). Activation of PI3K is also needed for LPS-induced IL-1β expression (Ojaniemi et al., 2003). PI3K activation is indispensable for LPS (Manna and Aggarwal, 2000) and CpG (Ishii et al., 2002)-induced activation of NF-κB. These reports indicated a positive regulatory role of PI3K in TLR signaling. In contrast, several lines of evidence suggest negative regulation by PI3K. LPS-induced NO production is increased in the presence of PI3K inhibitors (Diaz-Guerra et al., 1999). Gene targeting of the regulatory subunit of PI3K (p85α) and treatment with PI3K inhibitors resulted in augmentation of LPS-induced IL-12 production (Fukao et al., 2002). TRIF-dependent NF-κB activation and IFN-β synthesis are enhanced by PI3K inhibition (Aksoy et al., 2005). Most of these studies used pharmacological tools, wortmannin and LY294002, to inhibit PI3K. Although several studies have used mutants of PI3K and Akt to allow more specific analysis, most have determined the effects of the mutants by transcription factor reporter assay under artificial conditions. Here, we determined the effects of shRNA probe targeting p110β on TLR signaling in a mouse macrophage cell line. We also examined the effects of dominant-negative mutants of PI3K on TLR-mediated NO production in macrophages. The results indicated that PI3K is a negative regulator in TLR-mediated NF-κB activation. Pharmacological data with wortmannin supported the conclusion. In contrast, LY294002 inhibited the TLR signaling in a manner independent of its action on PI3K.

Materials and Methods

Reagents. Materials were obtained from the following sources: LPS (*Escherichia coli* serotype 0111: B4), bovine serum albumin (fatty acid-free), lipoteichoic acid (*Staphylococcus aureus*), and protein inhibitor cocktail were from Sigma (St. Louis, MO); IL-12p40 and IL-6 ELISA kits were from Genzyme (Minneapolis, MN) and GE

Healthcare (Little Chalfont, Buckinghamshire, UK), respectively; Griess Romijin Nitrite Reagent was from Wako (Tokyo, Japan); LY294002 was from Cayman Chemical (Ann Arbor, MI); wortmannin was from Kyowa Medex (Tokyo, Japan); peptidoglycan (*S. aureus*) was from Fluka (Riedel-deHaën, Switzerland); poly(I:C) was from Amersham Biosciences (Piscataway, NJ); CpG DNA (Cy3-oligonucleotide with the sequence TCC ATG ACG TTC CTG ATG CT) was synthesized by Hokkaido System Science (Sapporo, Japan); antibodies to phospho-Akt (Thr308), phospho-Erk1/2 (Thr202/Tyr204), and iNOS were from Cell Signaling Technology Inc. (Beverly, MA); anti-phospho-IκB (Ser32) was from Santa Cruz Biotechnology (Santa Cruz, CA); rGM-CSF was from PeproTech EC (London, UK); protein assay kit from Bio-Rad (Hercules, CA); and a synthetic lipopeptide based on the full-length macrophage-activating lipopeptide-2 kDa (Malp-2) was prepared with dipalmitoyl-*S*-glyceryl cysteine, as described previously (Nishiguchi et al., 2001).

Cells. Human macrophages were prepared from peripheral blood monocytes as described elsewhere (Nishiguchi et al., 2001). In brief, aliquots of 400 ml of citrate-phosphate dextrose-supplemented human blood were separated by methylcellulose sedimentation and density-gradient centrifugation with Ficoll-Paque to obtain peripheral blood mononuclear cells. Monocytes were isolated from these peripheral blood mononuclear cells with a magnetic cell-sorting system using anti-CD14-coated microbeads and cultured for 7 to 8 days in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and 50 IU/ml rGM-CSF. Raw264.7 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium fortified with glucose to a concentration of 4.5 g/l and 10% FCS. The cells were treated with or without inhibitors for 15 min except where otherwise specified and stimulated with various TLR ligands. 293T cells were maintained in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% FCS.

Plasmids. A plasmid encoding a dominant-negative mutant of bovine p85 (pCMV5-FLAG-Δp85) and the pH1 vector (Sasai et al., 2005) for expression of short hairpin RNAs was kindly provided by Dr. M. Kasuga (Kobe University, Kobe, Japan), and Dr. N. Inoue (Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan), respectively. A deletion mutant of p85cSH2 (1495-end) and catalytically active PI3K p110α subunit (p110αCAAT) were generated by polymerase chain reaction. Mammalian expression vectors containing N-terminal FLAG-tagged TLR2 and TLR6 were from Dr. T. Seya (Hokkaido University, Sapporo, Japan).

Cytokine Assay. Untreated human macrophages or those treated with inhibitors for 15 min in 96-well plates (5×10^4 cells/well) were stimulated with LPS, peptidoglycan, or Malp-2 for 18 h. The concentrations of IL-12p40 and IL-6 in the supernatants were measured by ELISA.

Western Blotting. Raw264.7 cells in 24-well plates were treated with inhibitors or stimuli and lysed in 50 μl of lysis buffer containing 25 mM Tris-HCl, pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM sodium orthovanadate (Na$_3$VO$_4$), 1 mM EDTA, 0.1% bovine serum albumin, 20 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 20 μM *p*-amidinophenylmethylsulfonyl fluoride, and 1 mM *N*-ethylmaleimide. The cell lysates were centrifuged at 15,000 rpm for 10 min. Proteins in the resultant supernatants were determined using a Bio-Rad assay kit. Total cell lysates (100 μg of protein) were mixed with 10 μl of 5$\times$ sample buffer (62.5 mM Tris, pH 6.8, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.02% bromphenol blue as final concentrations), and heated at 100°C for 3 min. The proteins were separated by SDS-PAGE and transferred electrophoretically onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked in 5% skim milk and incubated with appropriate antibodies. The associated antibodies were located by enhanced chemiluminescence (PerkinElmer, Norwalk, CT).

Nitric Oxide Production. Raw264.7 cells in 96-well plates were treated with inhibitors and were stimulated with various TLR li-

gands. The culture supernatants (50 or 100 μl) were transferred to 96-well assay plates, mixed with Griess reagent, and incubated for 15 min at room temperature. The absorbance (520 nm) of the reaction in each well was measured using a plate reader.

RNA Isolation and RT-PCR. Total RNA was isolated with an RNeasy kit (QIAGEN, Hilden, Germany). Single-stranded cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase. The cDNAs for iNOS, β-actin, and IFN-β were amplified by a polymerase chain reaction-based method using the following specific primers: iNOs, (sense) 5′-TGGGAATGGAGACTGTC-CCAG-3′ and (antisense) 5′-GGGATCTGAATGTGATGTTTG-3′; β-actin, (sense) 5′-atcatgtttgagaccttcaacacc-3′ and (antisense) 5′-gat-gtcacgcacgatttccc-3′; IFN-β, (sense) 5′-TTCCTGCTGTGCTTCTC-CAC-3′ and (antisense) 5′-GATTCACTACCAGTCCCAGAGTC-3′.

Reporter Gene Assay for NF-κB. Human embryonic kidney 293T cells in 24-well plates were transfected with 25 ng of NF-κB reporter plasmid (Stratagene, La Jolla, CA), 2 ng of sea pansy luciferase reporter plasmid (pRL-SV40 vector; Promega, Madison, WI) as an internal control, along with 2 ng of TLR4 and 0.3 ng of MD-2 using FuGENE 6 (Roche, Indianapolis, IN). Twenty-four hours after transfection, cells were treated with or without wortmannin for 15 min and stimulated with LPS for 6 h. Luciferase activity in the cell lysate was measured with Dual-Luciferase Reporter Assay System (Promega).

RNA Interference. Two sequences, 5′-gaagcagccgtgttattat-3′ and 5′-GCAGGGATTAAAGTGTGAA-3′, were selected to interfere with p110β expression. For each of these sequences, a pair of oligonucleotides were synthesized with sequences 5′-CCC(X)$_{19}$TTCAAG-AGA(Y)$_{19}$TTTTTGGAAA-3′ and 5′-CTAGTTTCCAAAAA(Y)$_{19}$TCTC-TTGAA(X)$_{19}$GGGTGCA, where (X)$_{19}$ is the coding sequence and (Y)$_{19}$ is the complementary sequence. The oligonucleotide pairs were annealed and ligated downstream of the H1-RNA promoter at the PstI and XbaI sites of the pH1 vector. Both vectors were transfected into Raw264.7 cells (5 × 10^6 cells) by electroporation in a final volume of 300 μl at 250 V/950 μF (Gene Pulser II; Bio-Rad).

Results

Effects of PI3K Inhibitors on TLR-Mediated IL-6 Production in Human Macrophages. Human monocyte-derived macrophages were treated with increasing concentrations of wortmannin or LY294002 followed by stimulation with TLR ligands, and the accumulation of IL-6 in the culture medium was determined. As shown in Fig. 1, a TLR4 ligand, LPS, markedly increased the level of IL-6 production by the cells. Wortmannin augmented the effect of LPS in a dose-dependent manner with an EC$_{50}$ value of approximately 50 nM (Fig. 1A). In contrast, another inhibitor of PI3K, LY294002, completely inhibited the TLR4-mediated

(Fig. 1B). TLR2 ligands, Malp-2 and peptidoglycan, were effective in inducing IL-6 production by the cells, although their effects were weaker than those of LPS. The TLR2-mediated IL-6 production was again markedly increased in the presence of wortmannin (Fig. 1A). LY294002 completely suppressed the TLR2-mediated cytokine production (Fig. 1B). Similar results were obtained when the effects of the PI3K inhibitors on the TLR2- or TLR4-mediated IL12p40 production were examined (data not shown).

Effects of PI3K Inhibitors on LPS-Induced iNOS Expression in Mouse Macrophages. The mouse macrophage cell line, Raw264.7, was treated with wortmannin, stimulated with various concentrations of LPS, and the expression of iNOS was determined by Western blotting (Fig. 2A). Wortmannin markedly enhanced the LPS-induced iNOS expression. In the presence of 0.3 μM wortmannin, LPS-induced iNOS production saturated at LPS concentrations higher than 10 ng/ml. Wortmannin also enhanced LPS-induced nitrite accumulation well (Fig. 2B). Thus, wortmannin seemed to enhance TLR-mediated events in both human (Fig. 1) and mouse macrophages. The enhancing effect of wortmannin was prominent when lower concentrations of LPS were used (Fig. 2B). However, the effect was minimal at higher concentrations of LPS, because accumulation of nitrite saturated at 10 ng/ml LPS. Therefore, in subsequent experiments, we used a suboptimal concentration of LPS to examine the enhancing effect of wortmannin.
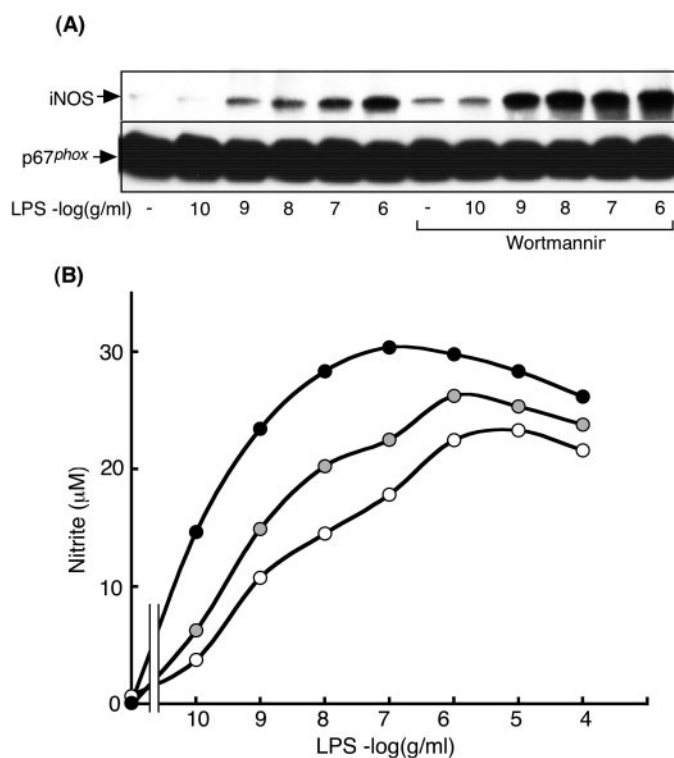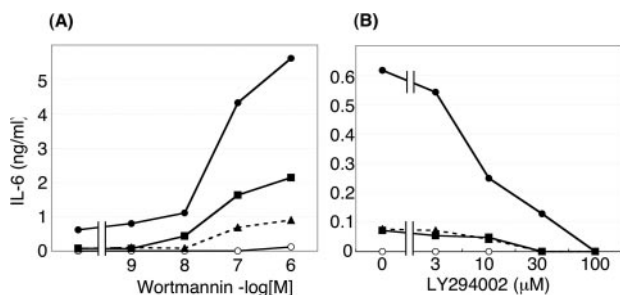

Fig. 1. Effects of PI3K inhibitors on TLR-mediated IL-6 production. Human macrophages were treated with increasing concentrations of wortmannin (A) or LY294002 (B) for 15 min followed by stimulation with 50 ng/ml LPS (●), 0.1 μg/ml Malp-2 (■), 5 μg/ml peptidoglycan (▲), or vehicle (○) for 18 h. The culture supernatants were collected, and the IL-6 levels were determined by ELISA.

Fig. 2. Enhancement by wortmannin of LPS-induced nitrite production. Raw264.7 cells were untreated or treated with wortmannin for 15 min followed by stimulation with increasing concentrations of LPS. A, the amounts of iNOS after 6-h treatment with LPS were determined by Western blotting with specific antibody (top). The membrane used for the blot was reprobed with anti-p67^{phox} antibody to establish that equal amount of cellular protein had been applied on SDS-PAGE (bottom). The concentration of wortmannin was 0.3 μM. B, nitrite accumulation in the supernatants after 20-h treatment with LPS were determined using Griess reagent. The concentrations of wortmannin were 0 (○), 0.1 (◐), or 1 μM (●).

Effects of Wortmannin on iNOS Expression Induced by Various TLR Ligands. The potentiation of iNOS expression by wortmannin was observed when macrophages were stimulated with peptidoglycan (TLR1 and 2), Malp-2 (TLR2 and 6), (poly)I:C (TLR3), lipoteichoic acid (TLR4), or CpG (TLR9) (Fig. 3). These TLRs associate with different adaptors; TLR1, with MyD88; TLR2, with MyD88/Mal; TLR6, with MyD88; TLR3, with TRIF; TLR4 with MyD88/Mal/TRIF/TIRAM; and TLR9, with MyD88. Therefore, it is very likely that the negative feedback regulation by PI3K is common to most TLRs. PI3K may modify the function of a protein component common to all of the adaptors.

Effects of Coexistence of Wortmannin and LY294002 on LPS-Induced iNOS Expression. The inhibitory effect of LY294002 was obvious in LPS-induced iNOS expression in Raw264.7 cells (Fig. 4A, middle), indicating that the inhibition was also common to human and mouse macrophages. Even when the cells were activated by both LPS and wortmannin, LY294002 effectively inhibited iNOS expression (Fig. 4A, bottom). It is likely that the inhibitory action of LY294002 and the opposite action of wortmannin are exhibited via independent signaling pathways. We also speculated that the target molecule of LY294002 functions downstream of the target of wortmannin, because the effect of wortmannin was abolished completely in the presence of LY294002. LPS-induced accumulation of phosphorylated Akt (pAkt) and the basal pAkt was similarly inhibited by these PI3K inhibitors despite their opposite effects on iNOS expression (Fig. 4B).

Effects of PI3K Inhibitors on mRNA Synthesis Induced by LPS. In agreement with iNOS protein expression, LPS-induced iNOS mRNA expression was markedly increased by wortmannin and was inhibited by LY294002 (Fig. 5A). Similar results were obtained in experiments to examine LPS-induced expression of IFN-β mRNA (Fig. 5B). It has been suggested that both MyD88-dependent and TRIF-dependent pathways result in the activation of NF-κB, whereas only the TRIF-dependent pathway is able to activate IFN-β gene expression. Because wortmannin enhanced IL-6, IL-12, iNOS, and IFN-β production on LPS challenge, the inhibitor could modify both MyD88-dependent and TRIF-dependent pathways. LY294002 inhibited the TRIF-dependent increase in IFN-β mRNA (Fig. 5B, left) and other TLR-mediated responses.

Enhancement of TLR-Mediated iNOS Expression by PI3K Mutants. We next investigated the effects of dominant-negative mutants of PI3K to elucidate the role of PI3K in TLR signaling. Both p110α and p110β are known to be complexed with the common regulatory subunit, p85, the absence of which markedly impairs the stability of the catalytic subunits in cells (Yu et al., 1998; Brachmann et al., 2005). Binding of tyrosine-phosphorylated proteins to SH2
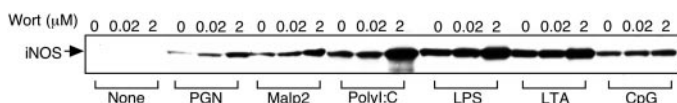
domains in p85 is one of the mechanisms leading to p110 activation. It has been demonstrated in several cell lines that Δp85, a mutant p85 regulatory subunit lacking the binding site to p110, inhibits class Ia PI3K-dependent cellular events
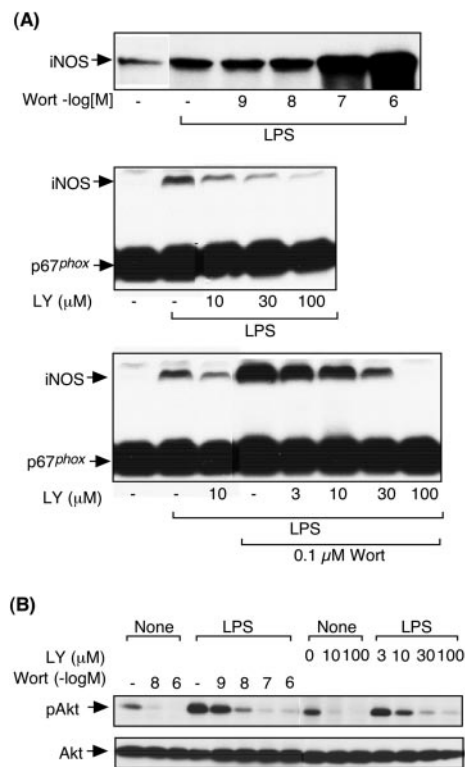

Fig. 4. Effects of PI3K inhibitors on LPS-induced iNOS expression. Raw264.7 cells were treated with various concentrations of wortmannin (Wort) and/or LY294002 (LY) for 15 min followed by stimulation with 50 ng/ml LPS. A, expression of iNOS after 6-h stimulation with LPS was determined by Western blotting with anti-iNOS antibody. Anti-p67^{phox} antibody was used for loading control. B, pAkt (top) after 15-min stimulation with LPS was determined using anti-phospho-Akt antibody. Also shown is the total amount of Akt (Akt, bottom) determined by Western blotting with anti-Akt antibody.
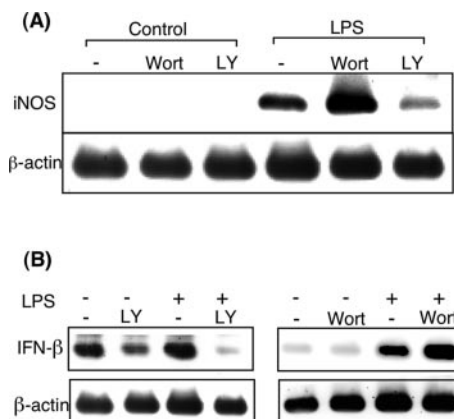

Fig. 5. Effects of PI3K inhibitors on LPS-induced expression of iNOS mRNA and IFN-β mRNA. A, Raw264.7 cells were treated with 0.1 μM wortmannin (Wort), 30 μM LY294002 (LY), or vehicle (−) for 15 min followed by stimulation with 0.1 μg/ml LPS for 6 h. Total RNA was extracted, and RT-PCR analysis was performed with the primers described under *Materials and Methods*. B, Raw264.7 cells were treated with the inhibitors as in A followed by stimulation with 0.1 μg/ml LPS (left) or 10 ng/ml LPS (right) for 6 h. Total RNA was extracted, and RT-PCR with 25 cycles was performed in the left and with 20 cycles in the right.

Fig. 3. Enhancements of iNOS production by various TLR ligands. Raw264.7 cells were treated with the indicated concentrations of wortmannin (Wort) followed by stimulation with 5 μg/ml peptidoglycan (PGN), 0.2 μM Malp-2, 50 μg/ml (poly)I:C, 10 ng/ml LPS, 5 μg/ml lipoteichoic acid (LTA), or 0.1 μM CpG DNA for 6 h. Total cell lysates were analyzed by Western blotting with anti-iNOS antibody.

(Hara et al., 1994). Likewise, a truncated form of p85 (p85cSH2; 1495 end), possessing the SH2 domain but lacking all other functional domains, is also expected to behave as a dominant-negative mutant. The mutants were prepared as FLAG-tagged constructs and were transfected into Raw264.7 cells by electroporation. Expression of the mutants was first checked using anti-FLAG antibody. The expression of p85cSH2 was easily detected (approximately 35 kDa; Fig. 6A), but the expression of Δp85 was hardly detectable when

(A) Total lysate   **(B)** P85 IP

Fig. 6. Increases in iNOS production by expression of dominant-negative mutants of PI3K or by depletion of p110β in LPS-treated cells. Raw264.7 cells ($5 \times 10^6/300$ μl) were transfected with 25 μg of FLAG-Δp85 (Δp85), 25 μg of FLAG-p85cSH2 (cSH2), or two shRNA constructs targeting p110β (15 μg each, Δp110β) by electroporation. Mock control (−) was subjected to electroporation with a similar condition. The cells were plated in 6- (A and B) or 24-well (C and D) plates and were cultured for approximately 40 h. A, total cell lysates were subjected to Western blotting with anti-FLAG antibody. B, aliquots of the lysate were immunoprecipitated with anti-p85 antibody and were subjected to Western blotting with anti-FLAG antibody (top) or anti-p110β antibody (bottom). C, the cells were incubated with or without 1 μM wortmannin (WT) for 15 min, followed by stimulation with 10 ng/ml LPS or 0.1 μM calyculin A (CA) for 15 min. Cell lysates were subjected to Western blotting with anti-pAkt, anti-p67^{phox}, or anti-phospho-Erk (pErk) antibodies. D, the cells were incubated with or without 1 μM wortmannin (WT) for 15 min, followed by stimulation with 10 ng/ml LPS for 6 h. Cell lysates were subjected to Western blotting with anti-iNOS antibody. E, the data in D were analyzed by a densitometer, and the density was plotted on the graph.

the whole-cell lysates were used for the assay. However, the expression of Δp85 was clearly detected when the lysates were subjected to immunoprecipitation with anti-p85 antibody, and the immune complex was examined with anti-FLAG antibody (Fig. 6B, top).

Transfection of Δp85 or p85cSH2 impaired the Akt activation induced by LPS (Fig. 6C, top). In contrast, the effect of calyculin A, a pan-inhibitor of serine/threonine phosphatases, was not susceptible to expression of the dominant-negative mutants of PI3K (Fig. 6C, top). Transfection of the empty vector did not cause any effects on the LPS-induced Akt phosphorylation (data not shown). The effects of the mutants were specific to the PI3K-dependent pathway in the sense that LPS-induced extracellular signal-regulated kinases 1 and 2 activation was not affected by the mutants (Fig. 6C, bottom). The amounts of p67^{phox} protein were almost unchanged among the samples, showing that equal amounts of cellular protein were applied to SDS-PAGE (Fig. 6C, bottom). Transfection of the dominant-negative PI3K resulted in enhancement of LPS-induced iNOS expression (Fig. 6, D and E). Wortmannin, which greatly enhanced the action of LPS in normal untransfected cells, produced no further increase in the effects of mutants. Similar results were observed in LPS-induced nitrite production (data not shown). It is likely that the endogenous PI3K was severely impaired even in the absence of wortmannin in cells transfected with the dominant-negative mutants. These results suggested that LPS-induced iNOS expression was suppressed by endogenous PI3K in normal cells.

The role of PI3K was further examined by the introduction of shRNA vectors targeting p110β to Raw264.7. The cell lysates were immunoprecipitated with anti-p85 antibody and were detected by Western blotting with anti-p110β antibody (Fig. 6B, bottom). The amount of p110β was decreased by the introduction of the shRNA vectors. Depletion of p110β resulted in enhancement of the LPS-mediated iNOS expression, as observed in cells transfected with the dominant-negative mutants (Fig. 6D). Again, the effect was not further increased by the addition of wortmannin (Fig. 6D). Thus, class Ia PI3K, at least p110β, plays a role in the negative regulation of TLR signaling.

Effects of Wortmannin on Stability of iNOS mRNA. To elucidate the mechanism by which PI3K inhibits the LPS-induced iNOS expression, wortmannin was added several times before and after LPS challenge (Fig. 7). Wortmannin was highly effective even when added 60 min before LPS. The effect decreased gradually when added after LPS challenge until almost no increase was observed. It is important to note that the enhancement was still remarkable when wortmannin was added several minutes after stimulation with LPS. It is likely that the inhibition of PI3K may not contribute to strengthening of the proximal signal of TLR. The inhibition of LPS-induced iNOS expression by LY294002 was also ob-
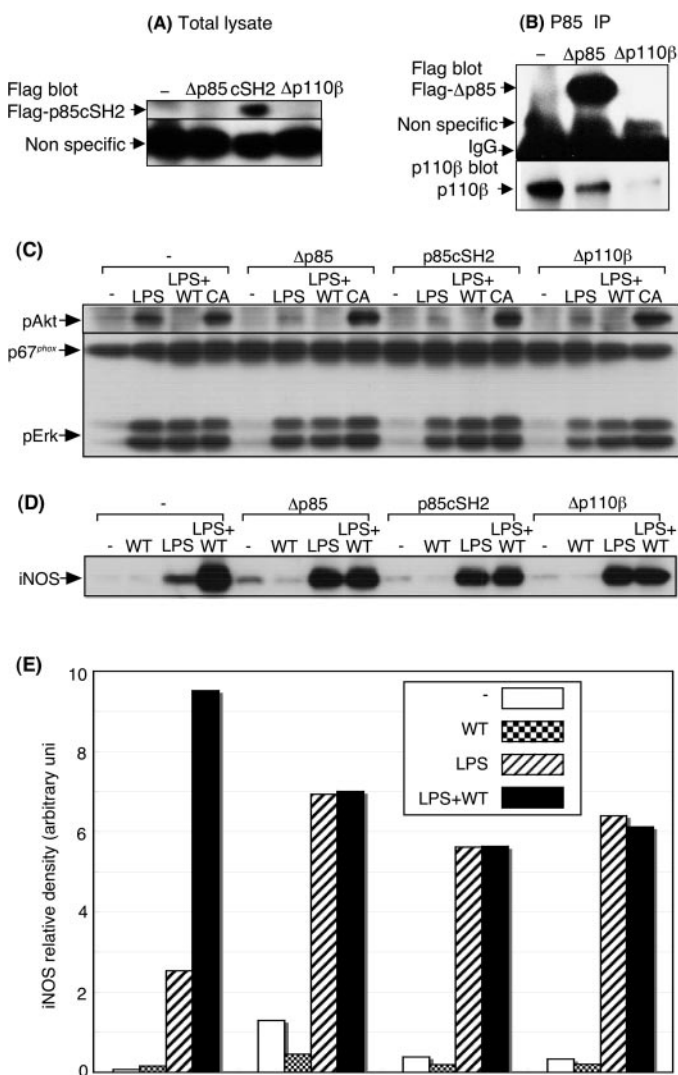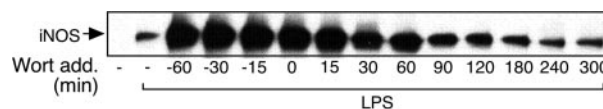

Fig. 7. Effects of wortmannin added at different time points with respect to LPS stimulation. Raw264.7 cells were incubated with 50 ng/ml LPS for 6 h before determination of the iNOS expression by Western blotting with anti-iNOS antibody. Wortmannin (WT, 0.1 μM) was added at various times before and after the addition of LPS.

served, even when the inhibitor was added 90 min after LPS addition (data not shown).

It has been reported that LPS increases cyclooxygenase-2 production, which is enhanced by the inhibition of PI3K (Monick et al., 2002). In this case, the inhibition of PI3K has been reported to stabilize cyclooxygenase-2 mRNA. Therefore, we next measured the rate of iNOS mRNA degradation with respect to wortmannin treatment. For these experiments, cells were first treated with or without wortmannin for 15 min, followed by the addition of LPS, and cultured for 6 h. At 6 h, actinomycin D was added to the culture medium, and the total RNA was harvested at 0 (without actinomycin), 6, and 12 h after addition. The initial level (time 0) of iNOS mRNA was higher in the wortmannin-treated cells (Fig. 8), but the degradation rate was the same regardless of the wortmannin treatment (Fig. 8). Thus, wortmannin-induced increases of mRNA levels were not a result of inhibition of degradation.

Effects of PI3K Inhibitors on NF-κB Activation. iNOS mRNA synthesis is increased by the activation of NF-κB. To examine the effect of wortmannin in NF-κB activation, we performed a reporter assay with 293T cells. The cells were transfected with TLR2, TLR6, and NF-κB-luciferase reporter plasmid and control plasmid. NF-κB-dependent induction of the luciferase reporter gene was activated 7-fold upon the addition of Malp-2, which was further increased in the presence of wortmannin (Fig. 9A). An NF-κB-independent expression of sea pansy luciferase from the control plasmid was unaffected at 0.1 μM wortmannin, but the expression was decreased by approximately 50% at 1 μM (data not shown). LY294002 impaired dramatically the transcriptional activity of the NF-κB reporter plasmid. The inhibitor was considered to possess a general inhibitory effect on promoter activities because it also impaired the transcriptional activity of NF-

κB-independent plasmids including phRL-TK, phRL-SV40, and phRL-NF-E2 (data not shown).

We also carried out reporter assays by overexpressing PI3K mutants. A dominant-negative form of PI3K, Δp85, increased Malp-2-induced and NF-κB-dependent induction of reporter gene (Fig. 9B, left), whereas catalytically active p110α subunit inhibited the NF-κB activation (Fig. 9B, right). The results supported the idea that PI3K negatively regulates TLR-mediated NF-κB activation.

Effects of PI3K Inhibitors on IκB Degradation. Activation of NF-κB is known to accompany the degradation of IκB, which binds NF-κB in the cytoplasm and thereby inhibits nuclear translocation of the transcription factor. Exposure of Raw264.7 to LPS led to the degradation of IκB, which was enhanced by wortmannin (Fig. 10). LY294002 also increased the IκB degradation. These observations support the suggestion that PI3K negatively regulates TLR-mediated IκB degradation because both LY294002 and wortmannin showed similar inhibitory effects on PI3K (Fig. 4B). The inhibitory effect of LY294002 on nitrite production and cytokine production (Figs. 1, 4, and 5) may be due to its effects on some protein(s) functioning downstream of the transcription factor.

Discussion

There is increasing evidence for the involvement of PI3K in TLR signaling. However, the role of PI3K is still unclear.
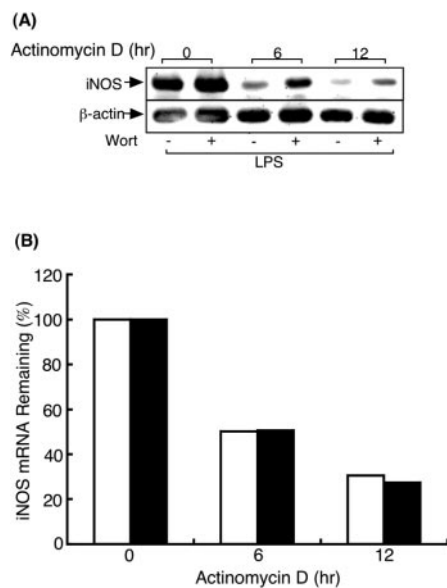
(A)

Fig. 8. Effects of wortmannin on iNOS mRNA stability. Raw264.7 cells were incubated with or without 0.1 μM wortmannin (Wort) for 15 min before stimulation with 10 ng/ml LPS for 6 h. The cells were then harvested or were incubated for a further 6 or 12 h with the addition of 0.5 μg/ml actinomycin D before harvest. A, the mRNA levels of iNOS and β-actin were evaluated by RT-PCR. B, the amounts of iNOS mRNA (iNOS mRNA/β-actin mRNA) in the wortmannin-treated (■) and untreated (□) cells are shown as percentages of the values at the time of addition of actinomycin D (0 h).
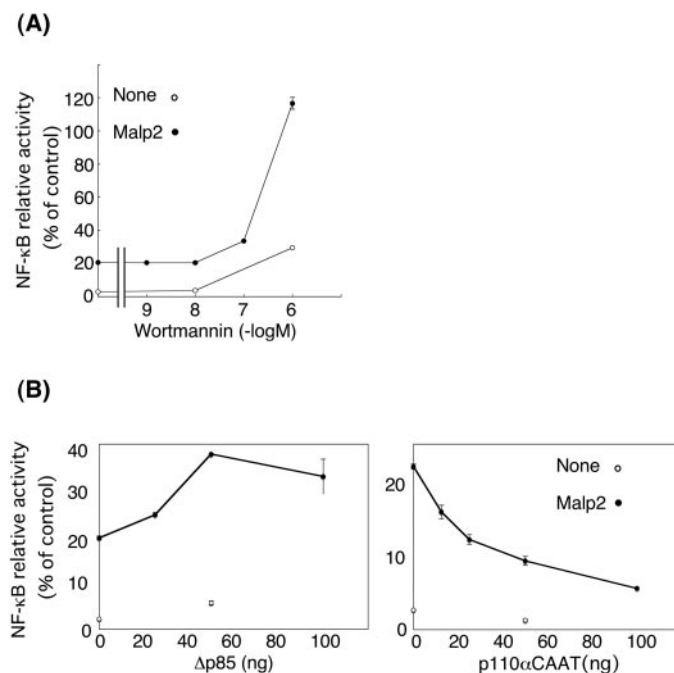
Fig. 9. Inhibition of PI3K activity enhances NF-κB promoter activity downstream of TLR. A, 293T cells were transfected with 25 ng of the NF-κB luciferase reporter gene plasmid and 2 ng of the sea pansy luciferase gene along with 10 ng each of TLR2 and TLR6. The following day, cells were treated with wortmannin for 15 min and stimulated with 0.1 μM concentration of Malp-2. After 6 h, cell extracts were prepared, and the expression levels of the firefly luciferase, which were normalized for those of control sea pansy luciferase, were determined. Results are the means ± S.D. of triplicate determinations. B, 293T cells were transfected with NF-κB luciferase, sea pansy luciferase, TLR2, and TLR6 as in A along with pCMV5-Δp85 (left) or pCMV5-p110αCAAT (right) for 6 h. The total amount of DNA transfected was kept constant by supplementation with control pCMV5. After 24 h, cells were stimulated with 0.1 μM Malp-2 for 6 h, and NF-κB luciferase activities were determined as in A.

Here, we presented direct evidence that PI3K, at least p110β, negatively regulates LPS-induced iNOS expression (Fig. 6). Our results using dominant-negative mutants of the regulatory subunit of PI3K also supported the suggestion that class Ia PI3K negatively regulates LPS-induced iNOS expression (Fig. 6). Pharmacological observations suggested that the negative feedback regulation by PI3K is common to most TLRs (Fig. 3).

Using pharmacological methods with dendritic cells and reporter assay with human embryonic kidney 293T cells, Aksoy et al. (2005) showed that inhibition of PI3K enhances TLR3- and TLR4-dependent NF-κB activation and IFN-β synthesis (Aksoy et al., 2005). They concluded that PI3K negatively regulates TRIF-dependent events because both TLR3 and TLR4 associate with TRIF to activate IFN-β synthesis. In the present study, we showed that inhibition of PI3K increased TLR2, -3, -4, and -9-mediated iNOS expression (Fig. 3). These receptors interact with different adaptors—MyD88/Mal, TRIF, MyD88/Mal/TRIF/TRAM, and MyD88, respectively. The results suggested that PI3K is involved in both MyD88-dependent and TRIF-dependent pathways. The conclusion was supported by the observations that IFN-β mRNA synthesis (Fig. 5B) and IL-6 (Fig. 1), IL-12p40 (data not shown), and iNOS expression were increased by wortmannin, in which IFN-β gene expression is entirely TRIF-dependent, and others were produced mainly through the MyD88-dependent pathway. There is another clue to elucidate the signaling pathway in which PI3K is involved. We showed that increased NF-κB activity in wortmannin-treated cells is the result of accelerated IκB degradation (Figs. 9 and 10). The increase in IκB degradation was dependent on the inhibition of PI3K because LY294002 exhibited a similar effect (Fig. 10). Therefore, we speculated that PI3K regulates protein(s) functioning upstream of IκB and commonly involved in the MyD88-dependent and TRIF-dependent pathways. Based on this consideration, TRAF6 or its associated proteins are possible candidates that may be regulated by PI3K. We showed that the enhancing effect of wortmannin could be observed even when added after LPS stimulation (Fig. 7). The result suggests that a putative target of wortmannin functions in the termination of the LPS signal. Because TRAF6 possesses E3 ubiquitin ligase activity, it is intriguing to consider that the degradation of a protein playing an indispensable role in TLR signaling is under the control of PI3K.

We observed the inhibition of various actions of LPS by LY294002: nitrite production and IFN-β mRNA synthesis in Raw264.7 cells, and IL-6 and IL-12p40 production in human macrophages. The inhibition was not based on the inhibition of PI3K, because dominant-negative mutants of PI3K and the shRNA probe targeting PI3K did not inhibit but rather increased the LPS-induced nitrite production (Fig. 6). These effects of the mutants and PI3K depletion were similar to those of wortmannin treatment. Because LY294002 antagonized the effect of wortmannin on TLR-mediated responses (Fig. 4), the inhibitor may modulate the target protein functioning downstream of PI3K. Our data are in good agreement with those of Kim et al. (2005), who reported that LY294002 inhibits the LPS-induced nitrite production of Raw264.7 cells by a mechanism independent of the inhibition of PI3K using LY303511, an inactive analog of LY294002. Contrasting with our data, they observed only a slight increase in LPS-induced iNOS expression by wortmannin. This seeming discrepancy may be because they used higher concentration of LPS to stimulate cells. We observed that the enhancing effect of wortmannin was minimized when the iNOS expression is fully stimulated by LPA (Fig. 2). Thus, we used submaximal concentration of LPS throughout this study.

The inhibition of TLR-mediated responses by LY294002 has been observed in many other studies (Arbibe et al., 2000; Ojaniemi et al., 2003; Dahle et al., 2004; Sarkar et al., 2004). In contrast, LY294002 and wortmannin have been reported to enhance TLR-mediated cell events, including cytokine production: IL-12p40 production in human monocytes (Martin et al., 2005), IFN-β production in human dendritic cells (Aksoy et al., 2005), cyclooxygenase-2 production in alveolar macrophages (Monick et al., 2002), and tumor necrosis factor-α production in peripheral blood mononuclear cells (Guha and Mackman, 2002). The reason for the discrepancy is currently unknown. However, because LY294002 increased the LPS-induced IκB degradation similarly to wortmannin (Fig. 10), the compound may enhance NF-κB activity under certain conditions. It is always difficult to estimate the specificity of inhibitors, especially when the effects of the inhibitors are determined by long-term incubation. We incubated the cells with the inhibitors for at least several hours to determine their effects on cytokine production or activation of transcription factors. The effects on autocrine/paracrine factors should be considered in addition to their nonspecific actions. In addition, some TLR ligands are particles or insoluble materials, which are incorporated into the cells by pinocytosis or phagocytosis at the first step. PI3K is indispensable for these processes regardless of the target particle (Ninomiya et al., 1994; Araki et al., 1996; Sun et al., 2003). Therefore, the role of PI3K in TLR signaling is further obscured in such cases; the function of PI3K in engulfment must be distinguished from that in TLR signaling.

In conclusion, our results suggest caution regarding the use of PI3K inhibitors in studying TLR signaling. It is very likely that PI3K is a negative feedback regulator of a wide variety of TLR responses, but further studies are needed to obtain a definitive answer regarding how PI3K is integrated into the system of TLR signaling.
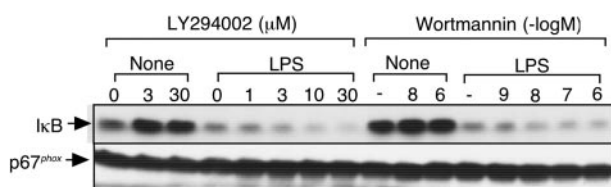
Fig. 10. Enhancement by PI3K inhibitors of LPS-induced IκB degradation. Raw264.7 cells were treated with increasing concentrations of LY294002 or wortmannin for 15 min followed by stimulation with 10 ng/ml LPS for 30 min. Total cell lysates were analyzed by Western blotting with anti-IκB antibody (top) or anti-p67^{phox} antibody (bottom).

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